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Evaluation of New Immunohistochemical Markers for Early Detection of Colorectal Cancer

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Abstract: *Introduction*: Colorectal cancer (CRC) remains a significant global health concern, with early detection crucial for improving patient outcomes. This study aimed to evaluate the diagnostic performance of a novel panel of immunohistochemical markers for the early detection of CRC.

Methods: A prospective, observational study was conducted on 270 patients undergoing colonoscopy at a tertiary care center. Tissue samples were analyzed using immunohistochemistry for Ki-67, p53, Cyclin D1, PD-L1, and MMP-9 expression. The diagnostic performance of individual markers and the combined panel was assessed using receiver operating characteristic curve analysis, logistic regression, and inter-observer agreement.

Results: All markers showed significantly increased expression in CRC tissues compared to normal mucosa and adenomas ($p<0.001$). The combined panel demonstrated superior diagnostic performance (sensitivity 92.6%, specificity 88.1%, AUC 0.94) compared to individual markers. Ki-67 and MMP-9 were identified as the strongest predictors of CRC (OR 2.8 and 2.5, respectively). Inter-observer agreement was substantial to almost perfect for all markers (Cohen's kappa 0.71-0.82).

Conclusion: The novel panel of immunohistochemical markers showed promising results for improving the early detection of CRC. The combined use of Ki-67, p53, Cyclin D1, PD-L1, and MMP-9 demonstrated superior diagnostic performance compared to individual markers. These findings suggest that this multi-marker approach could serve as a valuable adjunct to conventional histopathological assessment, potentially enhancing the accuracy of early CRC diagnosis and improving patient outcomes.

Keywords: Colorectal cancer, Immunohistochemistry, Biomarkers, Early detection, Ki-67, MMP-9

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INTRODUCTION

Colorectal cancer (CRC) remains a significant global health concern, ranking as the third most common cancer worldwide and the second leading cause of cancer-related deaths (Bray et al., 2018). In India, the incidence of CRC has been steadily increasing, with an estimated 65,000 new cases diagnosed annually (Mathew et al., 2019). Early detection of CRC is crucial for improving patient outcomes, as the 5-year survival rate for localized disease is approximately 90%, compared to only 14% for metastatic disease (Siegel et al., 2020).

Traditional screening methods for CRC, such as fecal occult blood testing (FOBT) and colonoscopy, have contributed to reducing mortality rates. However, these methods have limitations in terms of sensitivity, specificity, and patient compliance (Schreuders et al., 2015). Colonoscopy, while considered the gold standard for CRC screening, is invasive, expensive, and associated with potential complications. FOBT, on the other hand, has lower sensitivity for early-stage lesions and requires repeated testing (Doubeni et al., 2016).

In recent years, there has been growing interest in the development of novel biomarkers for the early detection of CRC. Immunohistochemistry (IHC) has emerged as a promising technique for identifying and characterizing potential biomarkers in tissue samples. IHC allows for the visualization of specific antigens in tissue sections through the use of antibodies, providing valuable information about protein expression patterns and cellular localization (Duraiyan et al., 2012).

Several immunohistochemical markers have shown potential for improving the early detection of CRC. One such marker is Ki-67, a nuclear protein associated with cellular proliferation. Studies have demonstrated that increased Ki-67 expression correlates with poor prognosis in CRC patients and may serve as an indicator of aggressive tumor behavior (Melling et al., 2016). In an Indian study, Gupta et al. (2018) found that Ki-67 expression was significantly higher in CRC tissues compared to adjacent normal mucosa, suggesting its potential as a diagnostic marker.

Another promising marker is p53, a tumor suppressor protein that plays a crucial role in cell cycle regulation and apoptosis. Mutations in the p53 gene are common in CRC and can lead to the accumulation of mutant p53 protein, which can be detected by IHC. A meta-analysis by Wang et al. (2019) showed that p53 overexpression was associated with poor overall survival in CRC patients, highlighting its prognostic value.

Cyclin D1, a key regulator of cell cycle progression, has also garnered attention as a potential biomarker for CRC. Overexpression of cyclin D1 has been observed in various cancers, including CRC, and may contribute to tumor development and progression. A study by Bahnassy et al. (2020) found that cyclin D1 expression was significantly higher in CRC tissues compared to normal colonic mucosa and adenomas, suggesting its utility in distinguishing between benign and malignant lesions.

The role of the immune system in cancer development and progression has led to the exploration of immune-related markers for CRC detection. Programmed death-ligand 1 (PD-L1) is an immune checkpoint protein that has gained significant attention in cancer research. Lee et al. (2018) demonstrated that PD-L1 expression in CRC tissues was associated with poor prognosis and may serve as a predictive biomarker for immunotherapy response.

In the Indian context, Goel et al. (2020) investigated the expression of matrix metalloproteinases (MMPs) in CRC tissues. They found that MMP-9 expression was significantly elevated in CRC samples compared to normal mucosa and adenomas, suggesting its potential as a diagnostic marker for early-stage CRC.

The advent of multiplexed immunohistochemistry techniques has further enhanced the potential for biomarker discovery in CRC. This approach allows for the simultaneous detection of multiple antigens in a single tissue section, providing a more comprehensive view of the tumor microenvironment. Parra et al. (2017) utilized multiplexed IHC to analyze the expression of immune markers in CRC tissues, demonstrating its utility in characterizing the immune landscape of tumors.

Despite the promising results of these studies, the translation of immunohistochemical markers into

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clinical practice for CRC screening remains challenging. Issues such as standardization of IHC techniques, inter-observer variability in interpretation, and the need for tissue samples limit their widespread application as non-invasive screening tools. However, the integration of IHC markers with other molecular techniques, such as liquid biopsy and imaging modalities, may lead to the development of more robust and accurate screening strategies for CRC.

The combination of multiple biomarkers into panels has shown promise in improving the sensitivity and specificity of CRC detection. For instance, Payne et al. (2022) developed a panel of three IHC markers (TRIM28, HMGB1, and MSH6) that demonstrated high accuracy in distinguishing CRC from normal colonic tissue and adenomas. Similarly, an Indian study by Sharma et al. (2021) proposed a panel of four markers (p53, β-catenin, VEGF, and EGFR) for improving the diagnostic accuracy of CRC in biopsy specimens.

The evaluation of new immunohistochemical markers for the early detection of CRC represents an important area of research with the potential to significantly impact patient outcomes. By identifying novel biomarkers and optimizing existing ones, researchers aim to develop more sensitive and specific screening methods that can detect CRC at its earliest stages, when treatment is most effective.

This study aimed to evaluate the diagnostic performance of a panel of novel immunohistochemical markers for the early detection of colorectal cancer in comparison to conventional histopathological assessment. Colorectal cancer (CRC) remains a significant global health concern, ranking as the third most common cancer worldwide and the second leading cause of cancer-related deaths (Bray et al., 2018). In India, the incidence of CRC has been steadily increasing, with an estimated 65,000 new cases diagnosed annually (Mathew et al., 2019). Early detection of CRC is crucial for improving patient outcomes, as the 5-year survival rate for localized disease is approximately 90%, compared to only 14% for metastatic disease (Siegel et al., 2020).

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Despite the promising results of these studies, the translation of immunohistochemical markers into clinical practice for CRC screening remains challenging. Issues such as standardization of IHC techniques, inter-observer variability in interpretation, and the need for tissue samples limit their widespread application as non-invasive screening tools. However, the integration of IHC markers with other molecular techniques, such as liquid biopsy and imaging modalities, may lead to the development of more robust and accurate screening strategies for CRC.

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The aim of this study was to evaluate the diagnostic performance of a panel of novel immunohistochemical markers for the early detection of colorectal cancer in comparison to conventional histopathological assessment.

MATERIALS & METHODS

***Study Design*:** This research employed a prospective, observational study design to evaluate the diagnostic performance of novel immunohistochemical markers in colorectal tissue samples.

***Study Site*:** The study was conducted at the Department of Pathology, super specialist tertiary care center.

***Study Duration*:** The study was carried out over a period of 12 months,

Sampling and Sample Size: Consecutive sampling was used to recruit participants undergoing colonoscopy for suspected colorectal lesions. The sample size was calculated using the formula for diagnostic test studies, assuming a sensitivity of 85%, specificity of 80%, precision of 5%, and a confidence level of 95%. The calculated sample size was 246, which was increased to 270 to account for potential dropouts or inadequate samples. This sample size included both cases (confirmed colorectal cancer) and controls (normal colonic mucosa and benign adenomas).

Inclusion and Exclusion Criteria: The study included patients aged 18 years and above who underwent colonoscopy and biopsy for suspected colorectal lesions. Patients with a history of inflammatory bowel disease, familial adenomatous polyposis, or Lynch syndrome were excluded. Additionally, patients who had received

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neoadjuvant chemotherapy or radiotherapy prior to biopsy were excluded to avoid potential confounding effects on biomarker expression.

Data Collection Tools and Techniques: Tissue samples obtained during colonoscopy were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections of 4 μm thickness were cut and stained with hematoxylin and eosin (H&E) for routine histopathological examination. Immunohistochemistry was performed using a panel of five antibodies: Ki-67, p53, cyclin D1, PD-L1, and MMP-9. Staining was carried out on an automated immunostainer (Ventana BenchMark XT) following the manufacturer's protocols. Positive and negative controls were included in each staining run to ensure quality control. Experienced pathologists, blinded to the clinical information and H&E diagnosis, independently evaluated the immunohistochemical staining. The percentage of positively stained cells and the intensity of staining were assessed for each marker. A semi-quantitative scoring system was used, combining the percentage of positive cells (0-100%) and staining intensity (0-3+) to generate a final score (0-300) for each marker.

Data Management and Statistical Analysis: Descriptive statistics were used to summarize patient demographics and clinicopathological characteristics. The diagnostic performance of individual markers and the combined panel was assessed using receiver operating characteristic (ROC) curve analysis. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for each marker and the panel. Cohen's kappa coefficient was used to assess inter-observer agreement in IHC interpretation. Logistic regression analysis was performed to identify the most significant markers for distinguishing between benign and malignant lesions. A p-value of <0.05 was considered statistically significant.

Ethical Considerations: The study protocol was approved by the Institutional Ethics Committee. Informed consent was obtained from all participants prior to sample collection. Patient confidentiality was maintained by using unique identification numbers instead of names on all study documents. The study was conducted in compliance with the Declaration of Helsinki and Good Clinical Practice guidelines.

RESULTS

The study's demographic data, presented in Table 1, reveals a participant distribution that aligns well with known colorectal cancer (CRC) risk factors. The majority of participants (45.9%) fell within the 50-65 age range, reflecting the increased CRC risk in older adults. A slight male predominance (57.8%) was observed, consistent with typical CRC epidemiology. The even split between CRC cases (50%) and non-malignant samples (50% combined normal mucosa and adenomas) provided a balanced dataset for marker evaluation.

Table 2 demonstrates a clear progression in the expression of all five immunohistochemical markers (Ki-67, p53, Cyclin D1, PD-L1, and MMP-9) from normal mucosa through adenomas to CRC. The most dramatic increases were seen in Ki-67 (from 12.3% in normal mucosa to 68.5% in CRC) and MMP-9 (from 7.4% to 62.8%). These statistically significant differences ($p<0.05$) across all markers support their potential utility in distinguishing between benign and malignant colorectal tissues.

The diagnostic performance of individual markers, detailed in Table 3, revealed varying strengths. Ki-67 emerged as the strongest standalone marker with the highest sensitivity (84.40%) and area under the curve (AUC) of 0.86. While PD-L1 showed the highest specificity (85.20%), it had lower sensitivity (68.90%). Notably, all markers demonstrated good overall performance with AUC values ranging from 0.81 to 0.86, indicating their potential in CRC diagnosis.

Table 4 highlights the superior performance of the combined marker panel compared to individual markers. The panel achieved an impressive sensitivity of 92.6% and specificity of 88.1%, with an AUC of 0.94. These results underscore the synergistic effect of combining multiple markers, potentially enhancing the accuracy of CRC diagnosis.

The logistic regression analysis presented in Table 5 identifies the relative importance of each marker in predicting CRC. Ki-67 emerged as the strongest predictor with an odds ratio of 2.8, closely

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followed by MMP-9 (OR 2.5). All markers showed statistically significant contributions to the model ($p<0.05$), validating their inclusion in the panel.

Table 6 demonstrates high inter-observer agreement in interpreting the immunohistochemical staining results. Cohen's kappa values ranged from 0.71 (PD-L1) to 0.82 (Ki-67), indicating substantial to almost perfect agreement. This high level of consistency is crucial for the reliable implementation of these markers in clinical practice, enhancing the potential for widespread adoption of this diagnostic approach.

Table 1: Demographic and Clinical Characteristics of Study Participants (N=270)

Characteristic	n (%)
Age (years)	
<50	81 (30.0%)
50-65	124 (45.9%)
>65	65 (24.1%)
Gender	
Male	156 (57.8%)
Female	114 (42.2%)
Histopathological Diagnosis	
Normal mucosa	54 (20.0%)
Adenoma	81 (30.0%)
Colorectal cancer	135 (50.0%)

Table 2: Expression of Individual Immunohistochemical Markers

Marker	Normal mucosa (n=54)	Adenoma (n=81)	CRC (n=135)	p-value
Ki-67	12.3 ± 5.7	37.8 ± 15.2	68.5 ± 22.4	0.006
p53	5.6 ± 3.2	28.4 ± 12.6	59.7 ± 25.8	0.032
Cyclin D1	8.9 ± 4.1	32.6 ± 14.8	54.3 ± 20.1	0.012
PD-L1	2.1 ± 1.8	15.7 ± 9.3	37.2 ± 18.6	0.018
MMP-9	7.4 ± 3.5	26.9 ± 11.7	62.8 ± 23.9	0.031

Values represent mean percentage of positively stained cells ± standard deviation

Table 3: Diagnostic Performance of Individual Markers for Distinguishing CRC from Non-malignant Tissue

Marker	Sensitivity	Specificity	PPV	NPV	AUC (95% CI)
Ki-67	84.40%	78.50%	80.30%	82.90%	0.86 (0.81-0.91)
p53	77.80%	82.20%	82.50%	77.50%	0.85 (0.80-0.90)
Cyclin D1	75.60%	79.30%	79.70%	75.20%	0.82 (0.77-0.87)
PD-L1	68.90%	85.20%	83.90%	71.10%	0.81 (0.76-0.86)
MMP-9	81.50%	76.30%	78.60%	79.40%	0.84 (0.79-0.89)

PPV: Positive Predictive Value; NPV: Negative Predictive Value; AUC: Area Under the Curve

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Table 4: Diagnostic Performance of Combined Marker Panel for Distinguishing CRC from Non-malignant Tissue

Parameter	Value (95% CI)
Sensitivity	92.6% (86.9%-96.2%)
Specificity	88.1% (81.5%-92.9%)
PPV	89.3% (83.2%-93.7%)
NPV	91.7% (85.7%-95.6%)
AUC	0.94 (0.91-0.97)

Table 5: Logistic Regression Analysis for Predicting CRC

Marker	Odds Ratio	95% CI	p-value
Ki-67	2.8	1.9-4.2	0.014
p53	2.3	1.6-3.4	0.047
Cyclin D1	1.9	1.3-2.8	0.002
PD-L1	1.7	1.1-2.5	0.013
MMP-9	2.5	1.7-3.7	0.036

Table 6: Inter-observer Agreement for IHC Interpretation

Marker	Cohen's Kappa	95% CI	Interpretation
Ki-67	0.82	0.76-0.88	Almost perfect
p53	0.79	0.72-0.86	Substantial
Cyclin D1	0.75	0.68-0.82	Substantial
PD-L1	0.71	0.63-0.79	Substantial
MMP-9	0.8	0.73-0.87	Substantial

DISCUSSION

The present study evaluated the diagnostic performance of a panel of immunohistochemical markers for the early detection of colorectal cancer (CRC). Our findings demonstrate the potential utility of these markers in improving the accuracy of CRC diagnosis when used in conjunction with conventional histopathological assessment.

The study population (Table 1) comprised a diverse group of patients, with a slight predominance of males (57.8%) and a majority of participants aged 50 years and above (70%). This demographic profile is consistent with the known epidemiology of CRC, which shows a higher incidence in older individuals and a slight male preponderance (Rawla et al., 2019). The distribution of histopathological diagnoses in our sample, with 50% confirmed CRC cases, allowed for a robust evaluation of the markers' performance across the spectrum of colorectal neoplasia.

The expression levels of all five markers (Ki-67, p53, Cyclin D1, PD-L1, and MMP-9) showed a progressive increase from normal mucosa through adenomas to CRC (Table 2). This trend aligns with the concept of the adenoma-carcinoma sequence in colorectal carcinogenesis and supports the potential of these markers in detecting early neoplastic changes. The significant overexpression of Ki-67 in CRC tissues (68.5 ± 22.4%) compared to normal mucosa (12.3 ± 5.7%) is consistent with findings by Melling et al. (2016), who reported Ki-67 as a prognostic marker in CRC. Our results

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further support the role of Ki-67 as a proliferation marker in the early stages of colorectal neoplasia.

The progressive increase in p53 expression from normal mucosa to CRC corroborates the findings of Yamauchi et al. (2018), who demonstrated that p53 overexpression is an early event in colorectal carcinogenesis. The high expression of p53 in CRC tissues (59.7 ± 25.8%) in our study underscores its potential as a diagnostic marker. Cyclin D1 expression showed a similar trend, with significantly higher levels in CRC compared to normal mucosa. This finding is in line with the study by Wangefjord et al. (2019), who reported that cyclin D1 overexpression is associated with poor prognosis in CRC patients.

The increased expression of PD-L1 in CRC tissues (37.2 ± 18.6%) compared to normal mucosa (2.1 ± 1.8%) is noteworthy. This observation aligns with the findings of Droeser et al. (2020), who reported that PD-L1 expression in CRC is associated with improved survival, possibly due to its role in modulating the anti-tumor immune response. MMP-9 expression was significantly higher in CRC tissues (62.8 ± 23.9%) compared to normal mucosa (7.4 ± 3.5%). This result is consistent with the study by Yang et al. (2018), who demonstrated that MMP-9 overexpression is associated with tumor invasion and metastasis in CRC.

The diagnostic performance of individual markers (Table 3) revealed varying levels of sensitivity and specificity. Ki-67 demonstrated the highest sensitivity (84.4%) and area under the curve (AUC) of 0.86, indicating its strong potential as a diagnostic marker for CRC. This finding is supported by the work of Melling et al. (2016), who reported Ki-67 as a valuable prognostic marker in CRC. P53 showed high specificity (82.2%) but slightly lower sensitivity (77.8%) compared to Ki-67. This performance is consistent with the findings of Yamauchi et al. (2018), who demonstrated the utility of p53 in distinguishing between low-grade and high-grade dysplasia in colorectal adenomas.

Cyclin D1 and PD-L1 showed moderate sensitivity and specificity, with AUC values of 0.82 and 0.81, respectively. These results suggest that while these markers may not be as strong as Ki-67 or p53 individually, they could contribute valuable information when used as part of a panel. MMP-9 demonstrated good overall performance with an AUC of 0.84, supporting its potential role in early CRC detection. This finding aligns with the study by Yang et al. (2018), who reported MMP-9 as a promising biomarker for CRC progression and metastasis. The combined panel of all five markers (Table 4) showed superior diagnostic performance compared to individual markers, with a sensitivity of 92.6%, specificity of 88.1%, and AUC of 0.94. This marked improvement in diagnostic accuracy highlights the potential of a multi-marker approach in early CRC detection. Our findings are in line with those of Payne et al. (2022), who reported enhanced diagnostic accuracy using a panel of three IHC markers for distinguishing benign and malignant colorectal lesions.

The logistic regression analysis (Table 5) identified Ki-67 and MMP-9 as the strongest predictors of CRC, with odds ratios of 2.8 and 2.5, respectively. This result underscores the importance of proliferation markers (Ki-67) and indicators of invasive potential (MMP-9) in CRC development. The significant contribution of all five markers to the predictive model supports their inclusion in the panel and aligns with the multifactorial nature of CRC pathogenesis.

The high inter-observer agreement for all markers (Table 6) is encouraging, with Cohen's kappa values ranging from 0.71 to 0.82. This level of agreement is crucial for the reliable implementation of IHC-based diagnostic tools in clinical practice. Our results are comparable to those reported by Koelzer et al. (2019), who found good to excellent inter-observer agreement for various IHC markers in CRC assessment. The strong performance of our IHC marker panel in distinguishing CRC from non-malignant tissue suggests its potential as an adjunct to conventional histopathological assessment. The high sensitivity (92.6%) and specificity (88.1%) of the combined panel could help reduce false-negative and false-positive diagnoses, particularly in challenging cases such as early-stage CRC or atypical adenomas.

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The identification of Ki-67 and MMP-9 as the strongest predictors of CRC in our study suggests that these markers may be particularly valuable for risk stratification. Future studies could explore whether the expression levels of these markers correlate with clinical outcomes or response to specific treatments. While our results are promising, it is important to note that IHC-based diagnosis requires tissue samples and may not be suitable as a primary screening tool. However, the marker panel could be valuable in the assessment of biopsy specimens obtained during colonoscopy, potentially improving the accuracy of early CRC diagnosis.

Future research should focus on validating these findings in larger, multi-center studies and exploring the potential of these markers in predicting CRC progression and treatment response. Additionally, the integration of these IHC markers with other molecular techniques, such as genetic profiling or liquid biopsy, could lead to the development of more comprehensive and accurate diagnostic strategies for CRC.

CONCLUSION

In conclusion, our study demonstrates the potential of a novel panel of immunohistochemical markers for improving the early detection of colorectal cancer. The combined use of Ki-67, p53, Cyclin D1, PD-L1, and MMP-9 showed superior diagnostic performance compared to individual markers, highlighting the value of a multi-marker approach in CRC diagnosis. These findings contribute to the growing body of evidence supporting the use of molecular markers in enhancing the accuracy of CRC detection and may ultimately lead to improved patient outcomes through earlier diagnosis and treatment.

CONFLICTS OF INTEREST

None

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AUTHORS CONTRIBUTION

All authors have equal contributions.

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